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A REFEREED PAPER

DETERMINATION OF GALACTURONIC ACID CONTENT OF PECTIN USING A MICROTITER PLATE ASSAY

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Abstract. **The amount of galacturonic acid residues in samples containing pectin is an important parameter in the quantitative and structural analysis of this complex polysaccharide. This paper describes a method to determine the content of galacturonic acids in samples containing pectin, using a glass microtiter plate and microtiter plate-reading equipment with standard interference filters. The assay is a modification of a procedure involving the hydrolysis of pectin in 80% sulfuric acid at 80 °C followed by a coloring step with 3,5 dimethylphenol reagent at room temperature. The previous assay was difficult to apply routinely if large numbers of samples were to be analyzed due to color changes in the assay that are time dependent. In addition the assay involves transferring of strongly acidic solutions to a cuvette prior to reading. The use of a microtiter plate assay has several practical advantages such as an accurate estimate of background absorbance by multiple reading of the plates, and many samples can be rapidly assayed in one plate to minimize errors due to fading of the chromophore. This method is particularly advantageous when a large number of pectin samples must be analyzed for their content of galacturonic acid residues and it minimizes the transfer of strongly acidic solutions.**

Pectin is a structural polysaccharide commonly found in the form of protopectin in plant cells. The backbone of pectin comprises in part of 1-4 linked galacturonic acid residues. An accurate method to assay for the content of galacturonic acid is important for the quantitative and structural analysis of these complex polysaccharides. Initial work was done (Dische, 1947, 1950) using a two-step heating procedure for hydrolysis and color development. The use of hot sulfuric acid in two steps resulted in brown products or "browning" by side reactions with neutral sugars. This procedure was later was modified (Blumenkrantz and Asboe-Hansen, 1973) using m-hydroxydiphenyl in the second stage of color development which reacted with uronic acids at room temperature. The room temperature step minimized browning, but browning still occurred in the first heating stage to some extent and had to be corrected for if neutral sugars were present during the assay. Another procedure was developed (Scott, 1979) using 3,5 dimethylphenol (DMP) which was claimed to be more specific for uronic acids by forming a chromophore within 10 min with a maximum absorbance of 450 nm. It was shown that neutral sugars reacting with DMP did not form a similar chromophore in the presence of chloride ion and corrections for browning could be made by subtracting the absorbance read at 400 nm. All of these procedures were typically done using a spectrophotometer equipped with standard 1 cm cuvettes and single readings were done sequentially using these instruments.

Neutral and uronic acid monosaccharides can be determined by ion-exchange chromatography using dilute sodium hydroxide and sodium hydroxide-sodium acetate solutions as eluants (Clarke et al., 1991; Lee, 1990) with a pulsed amperometric detector. Although these procedures are selective, they are difficult to perform on large numbers of samples as a typical chromatogram together with re-equilibration could take up to 90 min per sample to perform.

Spectrophotometers, that can read microtiter plates with 96 wells or greater, are available. The advantage of these microtiter plate spectrophotometers is that a large number of samples can be done simultaneously and the handling and transfer of hot, concentrated sulfuric acid is minimized relative to what is done using single-read spectrophotometers with 1 cm cuvettes. In addition the chromophore formed from reactions of phenolic compounds with uronic acids and neutral sugars may be unstable with a subsequent change in absorbance over time periods such as several minutes between readings. It is advantageous to take readings simultaneously in these reactions to minimize unwanted color development or bleaching that may occur between sample readings on conventional spectrophotometers, and that can introduce errors in the final reading. A microtiter plate assay for uronic acids had been developed (van den Hoogen et al., 1998) to measure hylauronic acid in synovial fluid, but a more specific microtiter plate method for galacturonic acid in pectin based on the conditions using 3,5 dimethylphenol (Scott, 1979) could be more accurate for assaying crude pectin fractions taken from plant tissue. Other microtiter procedures have been performed for hylauronic acid with carbazole but galacturonic acid had not been studied in this assay (Cesaretti et al., 2003).

Material and Methods

Glucose browning reaction before addition of DMP. The determination was performed in triplicate. To separate test tubes 0, 10, 25 and 50 µL of a 1% glucose (D-(+)-Glucose, SigmaUltra G7528-250G, Sigma-Aldrich, Inc., St. Louis, Mo.) solution was added. Deionized (DI) water was also added to bring the total volume up to 700 µL in each tube. Three mL of concentrated sulfuric acid (96.2% Baker Analyzed 9681-33, J.T. Baker, Inc., Phillipsburg, N.J.) containing 0.1% NaCl (S-9625 Sigma-Aldrich) was added to each tube individually and immediately vortexed for 15 sec (Vortex Genie 2—Model G-560, Scientific Industries, Inc., Bohemia, N.Y.). Each tube was then immediately placed on ice before transferring to solution basins. From each solution basin 240 µL was pipetted (12

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channel Finnpipette® S86019, Thermo Labsystems, Milford, Mass.) by column into the wells of the microtiter plate (Glass Microplate, Zinsser, Germany), which had been preheated to 80 °C in the block heater (VWR Standard Heat Block—13259-032) and allowed to heat for 15 min at 80 °C. The plate was removed and cooled in water at room temperature for 15 min. The plate was read at 400 and 450 nm using a microtiter plate reader (Power Wave 340 microtiter plate reader; Bio-Tek Industries, Highland Park, Winooski, Vt.).

Time reaction of glucose with DMP. Experimental conditions were the same as the study for browning reactions before addition of DMP, except 40 µL of DMP solution (0.2 g DMP (3,5-dimethylphenol 99%, 144134-5G, Sigma-Aldrich) in 100 mL of glacial acetic acid (A38-212, Fisher Chemical, Fair Lawn, N.J.) was added to each well in one row after reading the baseline at 400 and 450 nm. The microtiter plate reader was set to shake the plate at an intensity of 3 for 10 s before each read. Wells were read at time zero and 5 min intervals up to 30 min.

Time reaction of galacturonic acid with DMP. The determination was performed in triplicate. To separate test tubes 0, 100, 200, 300 and 400 µL of a 0.02% galacturonic acid (D-(+)-galacturonic acid, 48280, Fluka BioChemika GmbH, Steinheim, Switzerland) solution was added. DI water was added to bring the total volume up to 700 µL in each tube. The assay was performed as described for glucose reaction with DMP except 100 µL of DMP solution was added to each well.

Galacturonic acid standard curve with and without glucose added. The determination was performed in duplicate. To separate test tubes 0, 100, 200, 300, 400 and 500 µL of a 0.02% galacturonic acid solution was added. DI water was added to bring the total volume up to 700 µL in each tube. The assay was performed as described for the time reaction of galacturonic acid with DMP.

This procedure was repeated except 10 µL of a 1% glucose solution was added to each test tube in the initial step before adding the concentrated sulfuric acid.

Absorption spectra produced by reaction of glucose and galacturonic acid with 3,5 dimethylphenol. To separate test tubes 0, 10 µL of 1% glucose solution and 300 µL of a 0.02% galacturonic acid solution were added. DI water was added to bring the total volume up to 700 µL in each tube. The assay was performed as described for the time reaction of galacturonic acid with DMP.

After determining baseline values at 400 and 450 nm, 100 µL of DMP solution was added to the blank well on the plate and the plate was shaken for 10 s and the absorption spectrum was recorded at 10 nm intervals from 350 to 600 nm. This was repeated for the wells which contained the glucose and galacturonic acid samples. The spectrum was also recorded at 15 and 30 min after the addition of DMP for all these samples.

Result and Discussion

A goal of this study was to modify a previously described method (Scott, 1979) into a microtiter plate assay for galacturonic acid in pectin. This modified method should be safer and would minimize the handling of hot sulfuric acid and would allow for the simultaneous assay of a large number of samples to minimize errors due to chromophore bleaching or unwanted color development.

Intermediates can form from neutral sugars in hot concentrated H_2SO_4 and can react with each other or other species in solution to produce interfering absorbance or "browning". Neutral sugar interference can result in significant error when present at high concentrations during the assay of uronic acids such as galacturonic acid. Previous work (Scott, 1979) had shown that reducing neutral sugar interference, particularly from glucose, can be done by the use of 450-400 nm absorbance difference to measure uronic anhydride. The absorbance difference from glucose reaction products at 450 versus 400 nm could be used since the absorbance spectra for those species was nearly constant over that range. This was in contrast to the absorbance species from reaction of galacturonic acid with DMP that showed a maximum at 450 nm and this had been shown using procedures with 1 cm cuvettes.

A new experiment was designed to test similar conditions using a microtiter plate procedure. In the microtiter plate method, increasing concentrations of glucose were pipetted by columns into the microtiter plate. Before the addition of DMP the absorbance was read at both 400 and 450 nm and the average results were determined. As shown in Fig. 1, with 0 µg of glucose added, the initial absorbance at both 400 and 450 nm was 0.065 absorbance units. Increasing levels of glucose showed increasing absorbance values at both 400 and 450 nm as expected for browning and the values were similar within experimental error. The absorbance maximum, with 500 µg of glucose added, was 0.260 absorbance units at 450

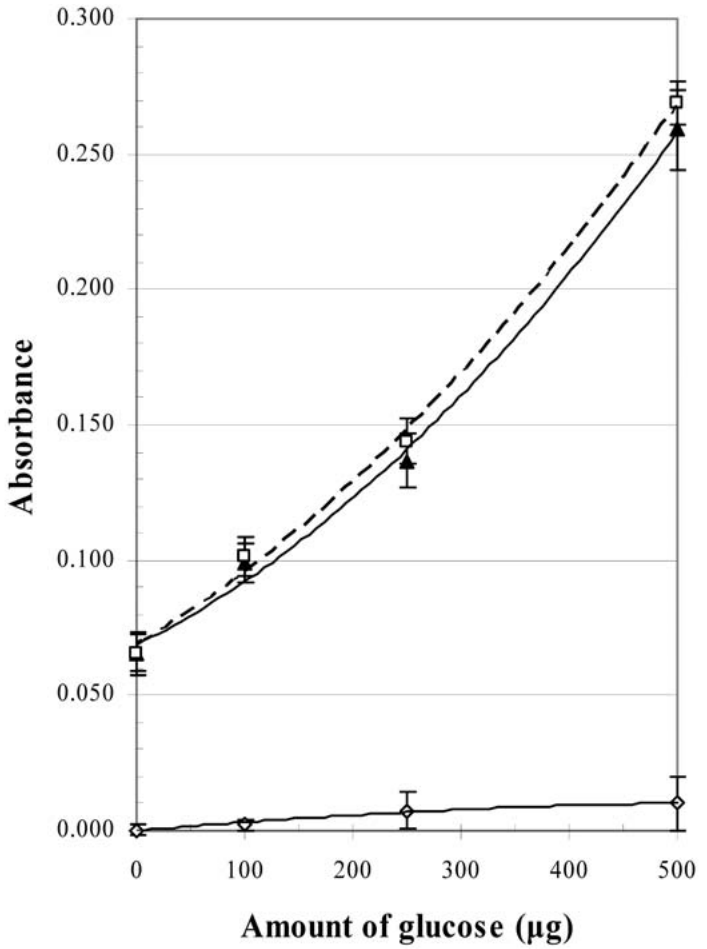

Fig. 1. Browning reaction of glucose with concentrated sulfuric acid and heat before addition of 3,5 dimethylphenol. —▲— Optical density at 400 nm, – –□– – Optical density at 450 nm, —◇— Difference in optical density at 450 versus 400 nm. Error bars represent standard error from triplicate runs.

nm. The difference in absorbance between 450 and 400 was less than 0.01 absorbance units even at 500 µg of glucose added to each well. These data indicate that the either the reading at 450 or 400 nm can be used to correct for browning reaction between concentrated sulfuric acid and glucose, but the 450 nm reading is preferred since it should result in less error. One advantage of the microtiter plate procedure is that a reading at 450 nm can be taken just prior to DMP addition to correct for browning, whereas with a standard spectrophotometer this is somewhat problematic since the reaction typically is not performed in the cuvette so the solution would have to be removed after blank reading to carry out the reaction and then reloaded to read at 450 nm. In addition these readings exhibited little or no change with time indicating stability of the chromophore (data not shown), nevertheless a blank reading with the microtiter plate procedure would be less than 20 sec for all 96 wells before DMP addition.

Prior to studying the galacturonic acid reaction, it was important to determine if glucose could produce a chromophore with DMP and if this reaction was time dependent. In this experiment the same plate was used as in the previous experiment (which contained increasing levels of glucose), and DMP was added to each row and then the absorbance increase was followed as a function of time. The time it took for the first reading, after DMP addition and mixing was performed, was approximately 20 s. Readings were recorded every 5 min for a total of 30 min of reaction time. The absorbance change for each level of glucose added is shown in Fig. 2. At time zero the readings were similar to those observed before DMP was added indicating that the absorbance at time zero was mostly due to browning. Of significance the absorbance at 450 nm increased with increasing time and was still increasing after 30 min of reaction. The rate of increase in absorbance corresponded to the level of glucose present. With 500 µg of glucose present the absorbance reached an absorbance value of 2.2 indicating the formation of a chromophore with DMP which was not a browning type reaction. This data demonstrates that glucose does form a chromophore with DMP and this product exhibits significant absorbance at 450 nm with chloride ion present. These findings would indicate that taking a reading at 450 nm immediately after DMP addition is important to minimize errors due to unwanted side reactions of non-galacturonic acid saccharides with DMP and the use of a microtiter plate reader facilitates the simultaneous rapid reading of multiple samples. These data are not consistent with previous work (Scott, 1979) where formation of chromophore between glucose and DMP was not observed when chloride ion was present.

The time zero readings as a function of the amount of glucose added after DMP addition was determined. Both absorbance at 400 and 450 nm was measured as a function of glucose concentration and are shown in Fig. 3. As expected, the absorbance increase with increasing glucose concentration mostly from the browning reaction, is similar to that observed in Fig. 1 with no DMP addition. Absorbance increases were similar for both 400 and 450 nm. The absorbance at 100 and 250 µg of glucose addition was similar to that observed with no DMP added. With the addition of 500 µg of glucose, the absorbance was 0.1 absorbance units higher at 450 nm after a DMP addition in comparison with no addition. This difference should be due to the reaction of glucose with DMP to form a chromophore in the 20 seconds before the absorbance reading could take place. This data indicates, that with high levels of non-galacturonic acid saccharides present, a rapid reading may not be sufficient to correct for the error due to unwanted reactions with DMP.

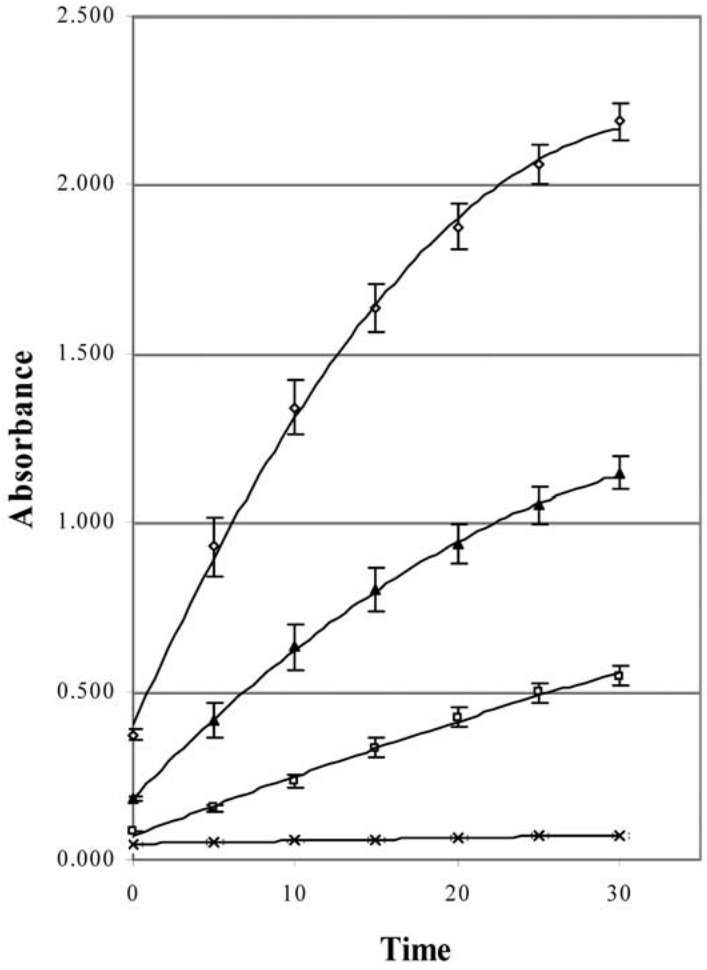

Fig. 2. Absorbance at 450 nm from reaction of 3,5 dimethylphenol and glucose as a function of time. —×— no glucose added, —□— 100 µg glucose added, —▲— 250 µg glucose added, —◇— 500 µg glucose added. Error bars represent standard error from triplicate runs and are smaller than symbol for sample with no glucose added.

To observe the kinetics of galacturonic acid with DMP a reaction was performed with increasing levels of galacturonic acid added as shown in Fig. 4. The first reading was performed within 20 sec of DMP addition for the whole plate. At galacturonic acid additions, from 20 to 60 µg, the maximum absorbance was reached by the time the first readings were taken. With 80 and 100 µg, the maximum was reached within the first five min and this maximum value was similar to the time zero value (100 µg data not shown on graph). After 5 min, all readings declined as the chromophore slowly "bleached" in the wells. This data indicates that the assay is under kinetic control in that taking rapid readings accounts for the chromophore formed between the galacturonic acid products and DMP within the first 20 s of reaction, whereas the chromophore produced from reactions between glucose and DMP are just beginning to form. A rapid reading, using this procedure, should produce accurate results if the level of contamination from non-galacturonic acid saccharides is limited, and a microtiter plate procedure with a simultaneous read would be best suited for this approach.

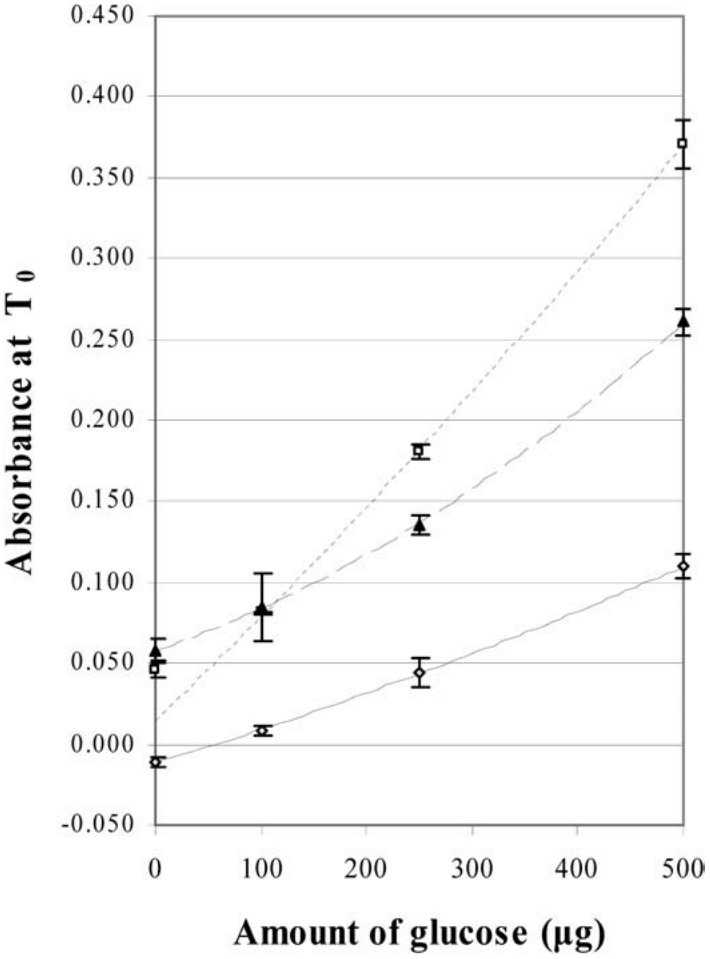

Fig. 3. Absorbance at time zero relative to glucose concentration after addition of 3,5 dimethylphenol. —▲— Optical density at 400 nm, --□-- Optical density at 450 nm, —◇— Difference in optical density at 450 versus 400 nm. Error bars represent standard error from triplicate runs.

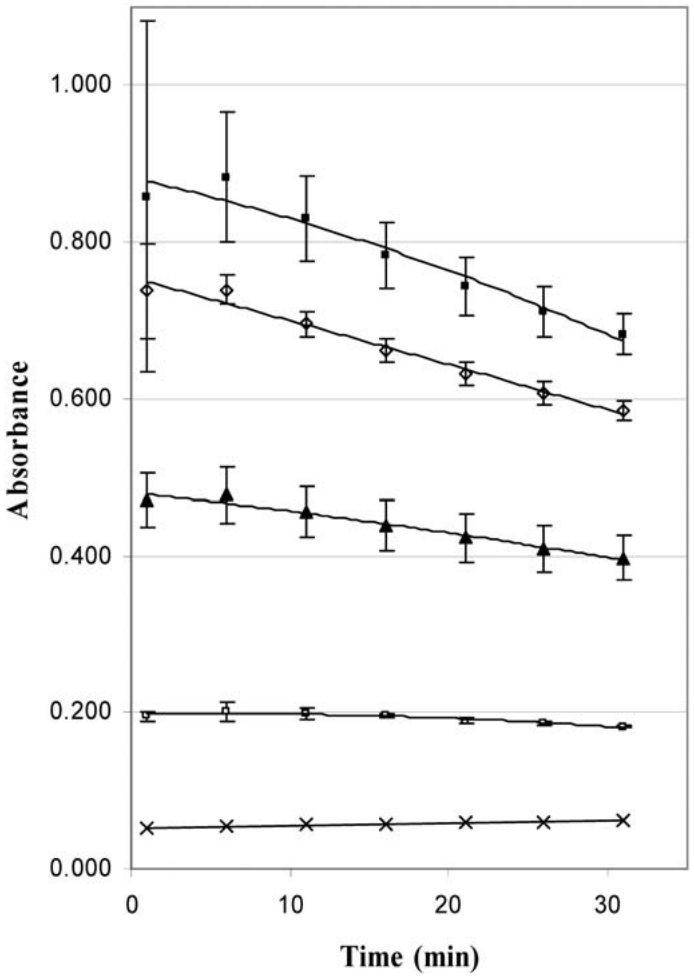

Fig. 4. Absorbance at 450 nm from reaction of 3,5 dimethylphenol and galacturonic acid as a function of time at different galacturonic acid concentrations. —×— no galacturonic acid added, —□— 20 µg galacturonic acid added, —▲— 40 µg galacturonic acid added, —◇— 60 µg galacturonic acid added, —■— 80 µg galacturonic acid added. Error bars represent standard error from duplicate runs and are smaller than symbol for 0 and 20 µg level.

To determine if a rapid absorbance reading of the reaction of DMP with galacturonic acid could produce results with minimal error with glucose addition, a standard curve for galacturonic acid was performed both in the presence and absence of glucose. All readings on the plate were done within 20 s of DMP addition to minimize reaction of DMP with glucose and are shown in Fig. 5. Without glucose addition the slope was 9.21×10^{-03} Abs/µg GA (R^2 = 0. 989) and with 100 µg glucose addition the slope was 8.89×10^{-03} Abs/µg GA (R^2 = 0.997). This resulted in a negative error on the slope of 3.3% on the standard curve with glucose added as compared to no glucose added. The relatively small error could be a result of pipetting error or could be due to interference from the presence of glucose. If glucose is producing a chromophore at 450 nm within 20 s, the expected error would have been positive. This data demonstrates that with a significant level of glucose added (approximately equal molar to the galacturonic acid) the effect on the galacturonic acid standard curve is minimal. Similar results have been observed with pectins using this assay (data not shown). This indicates that this assay could be of value in assaying pectins which have low levels of non-galacturonic acid saccharides present.

Interference could occur between neutral sugars and galacturonic acid during reaction with 3,5 dimethylphenol. Previous work indicated that glucose did not form a significant amount of chromophore at 450 nm and the 400 nm reading could be used to correct for browning (Scott, 1979). It had been shown previously that the presence of chloride ions during the assay minimized interference by neutral sugars (Scott, 1979). All assays done in the work reported here were done in the presence of chloride in an attempt to minimize interfering reactions from neutral sugars. An experiment was designed to determine the spectrum of the chromophore with glucose and DMP to determine if this product had a similar spectrum as that observed with galacturonic acid. In the experiment a spectral scan was performed separately with a blank containing no sugars, 60 µg of galacturonic acid, and 100 µg of glucose present in the assay. After mixing the spectrum was determined over a range of 350 to 600 nm at 10 nm intervals. The time to determine the complete spectrum was less than 30 sec.

As shown in Fig. 6, the absorption spectra produced by reaction of glucose and galacturonic acid with DMP are given. As expected, the blank reaction showed little or no chromophore being formed. In the reaction with galacturonic acid and DMP a peak with an absorption maximum at 450 nm is formed. This is the same absorption maximum and spec-

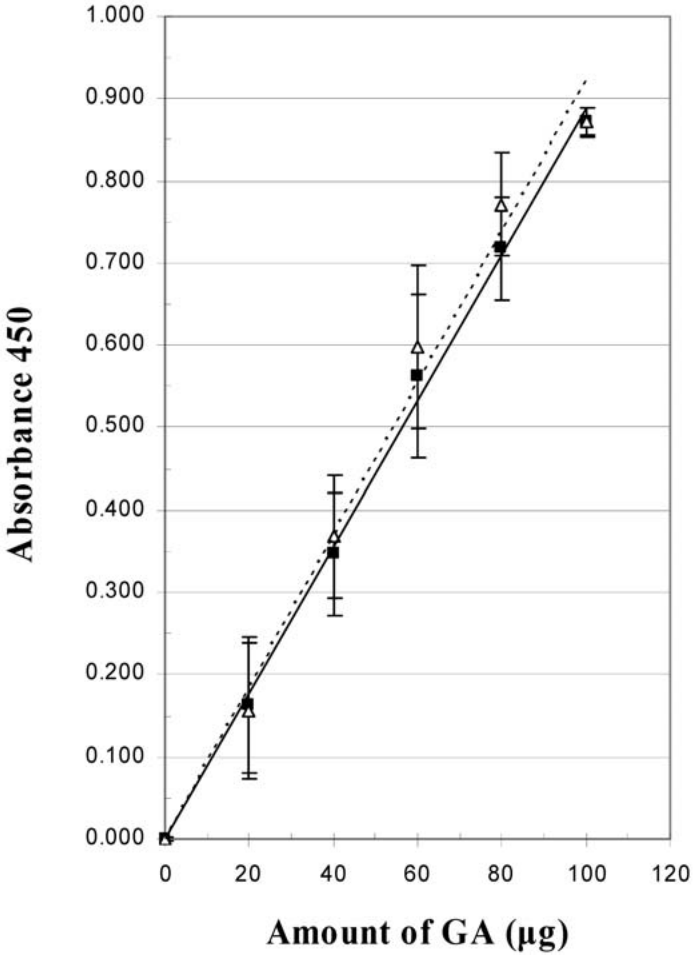

Fig. 5. Galacturonic acid standard curve with and without glucose added. --△-- galacturonic acid with no glucose added, —■— µg galacturonic acid with 100 µg of glucose added. Error bars represent standard error from duplicate runs.

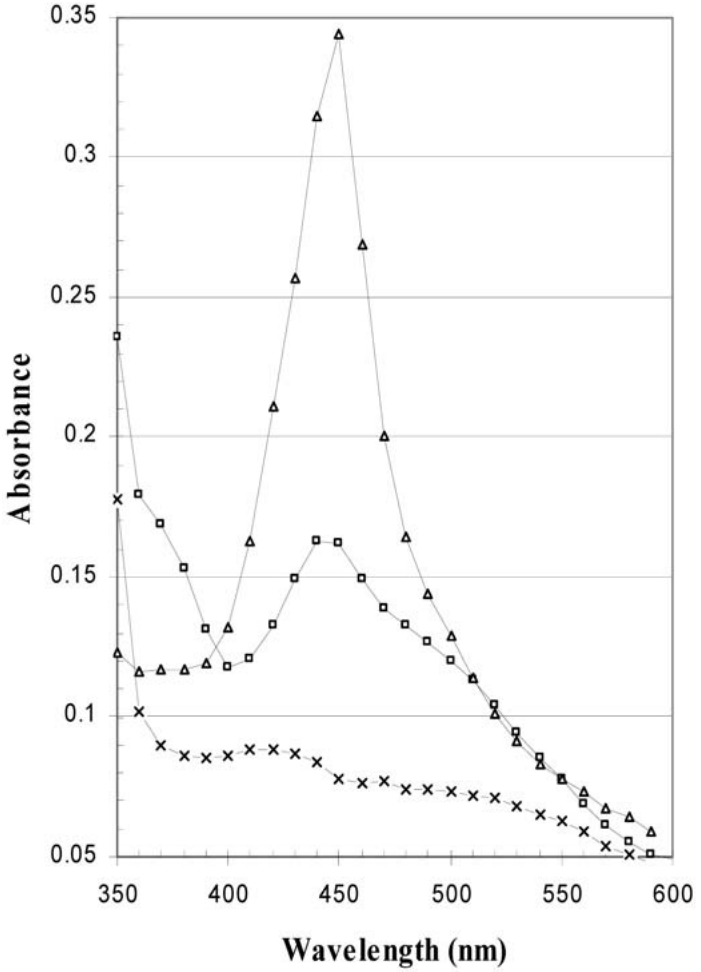

Fig. 6. Absorption spectra produced by reaction of glucose and galacturonic acid after addition of 3,5 dimethylphenol. —△— 40 µg galacturonic acid, —□— 100 µg glucose, —×— blank no saccharides present.

trum as reported previously and indicates that the chromophore being formed is similar to that previously reported (Scott, 1979). In the reaction with glucose a chromophore with an absorption maximum is also being formed early in the reaction with an absorption maximum of 450 nm and this is consistent with the kinetic results shown in Fig. 2. The shape of the spectra obtained with glucose and DMP after 30 min reaction time was similar to the initial spectra obtained except there was a significant increase in absorbance at 450 nm (data not shown). This data is not consistent with previous findings where it had been reported that glucose did not form a significant chromophore with DMP in the presence of chloride with a peak absorbance of 450 nm. This data does indicate the importance of performing a simultaneous rapid reading on a microtiter plate to minimize errors due to the presence of low levels of neutral sugars which may be present in pectin.

Conclusion

A microtiter plate assay has been developed for determining galacturonic acid and pectin content of extracts from plant tissue. Accurate measurements of galacturonic acid content can be determined if the presence of neutral sugars is limited. The reaction of DMP with galacturonic acid is rapid, less than 30 sec, whereas with neutral sugars, such as glucose, the color development takes approximately 15 min or more to reach maximum values. The reaction appears to be under kinetic control and accurate measurements of galacturonic acid require a rapid reading immediately upon DMP addition to minimize side reactions. The use of microtiter plates facilitates the rapid and simultaneous reading of multiple samples and minimizes errors due to unwanted side reactions with low concentrations of neutral sugars. Additional work will be required to minimize errors from side reactions due to high levels of neutral sugars which may be present in pectins.

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